APPLICATION

FOR

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TITLE: CONTINUOUS-FLOW METHOD FOR PREPARING
                MICROPARTICLES

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CONTINUOUS-FLOW METHOD FOR PREPARING MICROPARTICLES

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from U.S. Provisional Application Serial No. 60/166,516, filed on November 19, 1999.

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BACKGROUND OF THE INVENTION

This invention relates to a method for manufacturing polymeric microparticles.

Biodegradable synthetic polymers have been used as drug carriers. It has been observed that, when used as drug carriers, synthetic polymers are generally superior to natural polymers

10   with respect to reproducibility of product release (Okada et al., *Critical Reviews in Therapeutic Drug Carrier Systems* 12(1):1, 1995).

Several methods have been developed for preparing microspheres of hydrophobic synthetic polymers containing hydrophilic drugs. These methods include (1) emulsion solvent evaporation (e.g., oil/water, water/oil, water/oil/water emulsion evaporation), (2) phase

15   separation, (3) interfacial polymerization, and (4) spray drying (*ibid.*).

SUMMARY OF THE INVENTION

The invention is based on the discovery of a method for scalable, continuous flow production of a nucleic acid-containing microparticle that maintains the structural integrity of the associated nucleic acid and results in a microparticle having a purity suitable for introduction

20   into an animal (e.g., human) host. Microparticles prepared according to the continuous flow processes described herein can be used for delivery of a nucleic acid for gene therapy, antisense therapy, vaccination, treatment of autoimmune disease, and either specific or non-specific modulation of an immune response (e.g., via cytokine regulation). The microparticles can additionally be used to deliver nucleic acid encoding a protein or peptide useful in any type of

25   therapy.

Accordingly, in one aspect the invention features a scalable continuous process for preparing nucleic acid-containing microparticles. The process includes the steps of:
(a) providing a mixing chamber and a solvent removal device; (b) continuously supplying a first emulsion to the mixing chamber (i.e., where the first emulsion includes (i) an organic solution,

including a polymeric material and an organic solvent (e.g., dichloromethane, "DCM"), mixed with (ii) a first aqueous solution, including a nucleic acid); (c) continuously supplying a second aqueous solution, including a surfactant, to the mixing chamber; (d) continuously emulsifying the first emulsion and the second aqueous solution in the mixing chamber to form a second

5   emulsion, which includes nucleic acid, polymeric material, water, and organic solvent; (e) continuously transferring the second emulsion from the mixing chamber to the solvent removal device; and (f) removing the organic solvent from the second emulsion in the solvent removal device to form an aqueous suspension of nucleic acid-containing microparticles. The first emulsion, the second aqueous solution, or both further includes a stabilizer.

10   Alternatively, step (b) can be carried out where the nucleic acid is not in aqueous solution (e.g., where the nucleic acid is dry). An advantage of carrying out this step with dry nucleic acid is that doing so can lead to a high encapsulation efficiency (e.g., near 100%).

In some embodiments, steps (b) and/or (d) are carried out at a temperature between about 2°C and about 8°C.

15   The average residence time of the first emulsion and the second aqueous solution in the mixing chamber can be less than about 60 seconds (e.g., less than 60 seconds, less than 45 seconds, less than 30 seconds, less than 20 seconds, less than 15 seconds, less than 10 seconds, less than 5 seconds, less than 2 seconds, or less than 1 second). The average residence time of the second emulsion in the solvent removal device can be less than about 3 hours

20   (e.g., less than 3 hours, less than 2 hours, less than 1 hour, less than 30 minutes, or less than 15 minutes). For the purposes of this application, "average residence time" is defined as the flow rate to volume ratio.

The first aqueous solution and the second aqueous solution can be, for example, of essentially equal osmolarity, buffering capacity, and pH. The second aqueous solution can,

25   optionally, include polyvinyl alcohol (PVA) and/or a carbohydrate such as sucrose. For the purposes of this application, the term "essentially equal" denotes less than about a 10% difference of one parameter of the first solution from the same parameter of the second solution. Thus, "essentially equal osmolarity" means that the number of particles per unit volume are within about 10% of each other, while "essentially equal pH" means pH units within about 10%

30   other (e.g., pH 7.5 and 8.0 would be essentially equal pH) and "essentially equal buffering capacity" means less than about 10% difference in concentration of buffer components. For the

purposes of this application, "essentially equal" and "essentially identical" are interchangeable terms.

The polymeric material can be, for example, a synthetic, biodegradable polymer such as poly-lactic-*co*-glycolic acid, "PLGA". The ratio of lactic acid to glycolic acid in the PLGA can

5   be, for example, between about 1:2 and about 4:1 by weight (e.g., about 1:1), and the PLGA can have an average molecular weight in the range of, for example, about 6,000 to 100,000.

The stabilizer can be a single compound or a mixture of compounds (e.g., a carbohydrate such as sucrose and a buffer such as TRIS-EDTA, "TE"). The stabilizer can alternatively or additionally include a lipid. The lipid can be, e.g., a cationic lipid, an anionic lipid, or a

10   zwitterionic lipid, or may have no charge. Examples of lipids include cetyltrimethylammonium bromide (CTAB) and phospholipids, e.g., phosphatidylcholine. Specific examples include polyethylene glycol distearoylphosphatidyl ethanolamine (PEG-DSPE), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), taurocholic acid, glycocholic acid, capric acid, N-lauryl sarcosine, fatty acyl carnitine and vitamin D3. The microparticles may contain

15   one or more than one type of lipid, e.g., those lipids present in lecithin lipid preparations, and may also include one or more additional stabilizers.

The lyophilized or freeze-dried microparticles can, for example, have a residual organic solvent level of less than about 200 ppm (e.g., less than 200 ppm, less than 150 ppm, less than 100 ppm, less than 50 ppm, less than 20 ppm, or less than 10 ppm). Alternatively, microparticles

20   can be air-dried and can have about the same residual organic solvent levels.

In some embodiments, the process described above also includes the steps of: (g) providing a diafiltration apparatus (e.g., including a hollow fiber system); (h) diluting the aqueous suspension with an aqueous wash solution; (i) supplying the diluted aqueous suspension to the diafiltration apparatus; and (j) removing an aqueous waste solution (i.e., including at least

25   some of the wash solution of step (h)) from the diluted aqueous suspension in the diafiltration apparatus, to form a purified aqueous suspension that includes nucleic acid-containing microparticles. The process can further include some or all the steps of: washing the purifued aqueous suspension to form a suspension of washed mictroparitcles; concentrating the suspension of washed microparticles or the purified aqueous suspension in the diafiltration

30   apparatus, to form a concentrate; and transferring the concentrate into one or more vessels. The process can still further include the step of: (m) lyophilizing, freeze-drying, or air drying the

concentrate aqueous suspension in the one or more vessels, to form lyophilized, freeze-dried, or air-dried microparticles. Steps (h), (i) and (j) can optionally be carried out at a temperature of between about 2°C and about 8°C.

In other embodiments, the process described above also includes the steps of:

5   (g) contacting the aqueous suspension with a vibrating or non-vibrating fine-mesh screen (e.g., as in a fine mesh stainless steel screen contained within a Sweco device or a cartridge filter);

(h) filtering the aqueous suspension through the screen to remove at least some of each of the first and second aqueous solutions and to retain the microparticles on the screen; (i) washing the microparticles with at least one aqueous wash solution (e.g., water-for-injection, "WFI") to

10   produce washed microparticles; and (j) drying the washed microparticles to produce dried microparticles. The aqueous wash solution can optionally be at a temperature of, for example, about 2°C to about 8°C. The process can also include the step of contacting the washed microparticles with an excipient (e.g., prior to the drying step), and/or the step of transferring the dried microparticles into one or more vessels.

15   In the above processes, the mixing chamber can, for example, include a homogenizer. The solvent removal device can be a bioreactor. The second aqueous solution can be supplied to the mixing chamber at a flow rate of between 0.1 and 100 l/min (e.g., 0.1 to 20 l/min or 0.1 to 50 l/min).

The organic solvent can be removed from the second emulsion in the solvent removal

20   device, for example, by evaporation, by heating the second emulsion in the solvent removal device (e.g., to between 30°C and 55°C), by an extraction process, by applying a partial vacuum to the solvent removal device, or any combination of these methods. Alternatively or additionally, the removal of the organic solvent from the second emulsion in the solvent removal device can be facilitated by diluting the second emulsion in the solvent removal device.

25   For the purposes of this application, a "solvent removal device" is a device that accomplishes removal of the solvent from microparticles, but not necessarily from the fluid in which they are suspended. Examples of suitable solvent removal devices include a bioreactor, a tank (e.g. a hardening tank), a hollow fiber cartridge and a device that contain the microparticles and have water passed through it (e.g. a Sweco device).

30   In any of the processes described above, each of the steps can be carried out aseptically.

In any of the processes described above, at least about 50% (e.g., 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more) of the nucleic acid in the microparticles can be in the form of circular RNA molecules or supercoiled circular DNA molecules.

The average diameter of microparticles can be, for example, less than about 100 microns (e.g., less than 100 microns, less than 50 microns, less than 20 microns, between about 0.5 and about 2.5 microns, inclusive, or smaller), whether measured according to number average or volume average.

Biodegradable is used here to mean that the polymers degrade over time into non-toxic compounds that are cleared from the host cells by normal metabolic pathways. Generally, a biodegradable polymer will be substantially metabolized within one to three months after injection into a patient, and essentially completely metabolized within about two years.

Essentially identical in the context of a DNA or polypeptide sequence is defined here to mean differing no more than 25% from the naturally occurring sequence, when the closest possible alignment is made with the reference sequence and where the differences do not adversely affect the desired function of the DNA or polypeptide in the methods of the invention. The phrase *fragment of a protein* is used to denote some portion of the protein that is at least four residues in length, but less than the whole protein.

The determination of percent homology between two sequences is accomplished using the algorithm of Karlin et al., *Proc. Natl. Acad. Sci. USA* 87:2264-2268, 1990, modified as in Karlin et al., *Proc. Natl. Acad Sci. USA* 90:5873-5877, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., *J. Mol. Biol.* 215:403-410, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al., *Nucleic Acids Res.* 25:3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) should be used. *See* http://www.ncbi.nlm.nih.gov.

The peptide or polypeptide encoded by the nucleic acid can be linked to a targeting sequence. The term *targeting sequence* is used interchangeably with trafficking sequence and

refers to an amino acid sequence that causes a polypeptide to which it is fused to be transported to a specific compartment of the cell, e.g., the nucleus, the endoplasmic reticulum, the golgi apparatus, an intracellular vesicle, a lysosome, or an endosome.

5 In the embodiment where the expression product includes a peptide having a length and sequence which permit it to bind an MHC class I or II molecule, the expression product is typically immunogenic or immunosuppressive. The expression product can have an amino acid sequence that differs in sequence identity by up to 25% from the sequence of a naturally occurring peptide or protein recognized by a T cell, provided that it can still be recognized by the same T cell. Such variant peptides may function exactly as the naturally occurring counterpart,

10 or instead may act by altering the cytokine profile of the T cell (i.e., an "altered peptide ligand"). The differences between the expression product and the naturally occurring peptide can, for example, be engineered to increase cross-reactivity to pathogenic viral strains, to remove or alter amino acids that give the protein an undesirable function, or to increase HLA-allotype binding.

Examples of *expression products* include amino acid sequences at least 50% identical to

15 the sequence of an MHC class II binding fragment of myelin basic protein (MBP), proteolipid protein (PLP), invariant chain, GAD65, islet cell antigen, desmoglein, α-crystallin, or β-crystallin. Table 1 lists many proteins that are thought to be involved in autoimmune disease. For example, the fragments may be essentially identical to any one of SEQ ID NOS: 1-46, such as MBP residues 80-102 (SEQ ID NO: 1), PLP residues 170-191 (SEQ ID NO: 2), or invariant

20 chain residues 80-124 (SEQ ID NO: 3). Other examples of fragments are listed in Table 2.

Alternatively, the expression product can include an amino acid sequence essentially identical to the sequence of an antigenic portion of any of the tumor antigens listed in Table 3, such as those encoded by the human papilloma virus E1, E2, E6, and E7 genes, Her2/neu gene, the prostate specific antigen gene, the melanoma antigen recognized by T cells (MART) gene, or

25 the melanoma antigen gene (MAGE). Again, the expression product can be engineered to increase cross-reactivity.

TABLE 1: Autoantigens

	Disease	Associated Antigen	Notes
	Coeliac disease	α-Gliadin	a
5	Goodpasture's syndrome	Basement membrane collagen	a
	Graves' disease	Thyroid Stimulating Hormone (TSH) receptor	a
	Hashimoto's disease	Thyroglobulin	a
	Isaac's syndrome	voltage-gated potassium channels	b
	Insulin-dependent	Glutamic acid decarboxylase (GAD)	a
10	diabetes	Insulin receptor	a
		Insulin associated antigen (IA-w)	a
		Hsp	b
	Lambert-Eaton myasthenic syndrome (LEMS)	Synaptogamin in voltage-gated calcium channels	b
15	Multiple sclerosis	Myelin basic protein (MBP)	a
		Proteolipid protein (PLP)	a
		Myelin oligodendrocyte-associated protein (MOG)	a
		αB-crystallin	a
20	Myasthenia gravis	Acetyl choline receptor	a
	Paraneoplastic encephalitis	RNA-binding protein HuD	b
	Pemphigus vulgaris	"PeV antigen complex"	a
		Desmoglein (DG)	c
25	Primary biliary cirrhosis	Dihydrolipoamide acetyltransferase	b
		Pyruvate dehydrogenase complex 2 (PDC-E2)	d
	Progressive systemic sclerosis	DNA topoisomerase	a
		RNA polymerase	a
	Rheumatoid arthritis	Immunoglobulin Fc	a
30		Collagen	
	Scleroderma	Topoisomerase I	b
	Stiff-man syndrome	Glutamic acid decarboxylase (GAD)	a
	Systemic lupus erythematosus	ds-DNA	a
35	Uveitis	Interphotoreceptor retinoid-binding protein	b
		S antigen (rod out segment)	b

References:

a)   HLA and Autoimmune Disease, R. Heard, pg. 123-151 in HLA & Disease, Academic Press, New York, 1994, (R. Lechler, ed.)

40   b)   Cell 80:7-10 (1995)

c)   Cell 67:869-877 (1991)

d)   JEM 181:1835-1845 (1995)

TABLE 2: Class II Associated Peptides

	Peptide	SEQ ID NO:	Source Protein
	Peptide	**SEQ ID NO:**	**Source Protein**
	GRTQDENPVVHFFKNIVTPRTPP	1	MBP 80-102
5	AVYVYIYFNTWTTCQFIAFPFK	2	PLP 170-191
	FKMRMATPLLMQA	3	Invariant chain 88-100
	TVGLQLIQLINVDEVNQIV		
	TTNVRLKQQWVDYNLKW	4	Achr α 32-67
	QIVTTNVRLKQQWVDYNLKW	5	Achr α 48-67
10	QWVDYNL	6	Achr α 59-65
	GGVKKIHIPSEKIWRPDL	7	Achr α 73-90
	AIVKFTKVLLQY	8	Achr α 101-112
	WTPPAIFKSYCEIIVTHFPF	9	Achr α 118-137
	MKLGTWTYDGSVV	10	Achr α 144-156
15	MKLGIWTYDGSVV	11	Achr α 144-157 analog(I-148)
	WTYDGSVVA	12	Achr α 149-157
	SCCPDTPYLDITYHFVM	13	Achr α 191-207
	DTPYLDITYHFVMQRLPL	14	Achr α 195-212
20	FIVNVIIPCLLFSFLTGLVFY	15	Achr α 214-234
	LLVIVELIPSTSS	16	Achr α 257-269
	STHVMPNWVRKVFIDTIPN	17	Achr α 304-322
	NWVRKVFIDTIPNIMFFS	18	Achr α 310-327
	IPNIMFFSTMKRPSREKQ	19	Achr α 320-337
25	AAAEWKYVAMVMDHIL	20	Achr α 395-410
	IIGTLAVFAGRLIELNQQG	21	Achr α 419-437
	GQTIEWIFIDPEAFTENGEW	22	Achr γ 165-184
	MAHYNRVPALPFPGDPRPYL	23	Achr γ 476-495
	LNSKIAFKIVSQEPA	24	desmoglein 3 190-204
30	TPMFLLSRNTGEVRT	25	desmoglein 3 206-220
	PLGFFPDHQLDPAFGA	26	HBS preS1 10-25
	LGFFPDHQLDPAFGANS	27	HBS preS1 11-27
	FFLLTRILTI	28	HBS Ag 19-28
	RILTIPQSLD	29	HBS Ag 24-33
35	TPTLVEVSRNLGK	30	HSA 444-456
	AKTIAYDEEARR	31	hsp 65 2-13
	VVTVRAERPG	32	hsp 18 61-70
	SQRHGSKYLATASTMDHARHG	33	MBP 7-27
	RDTGILDSIGRFFGGDRGAP	34	MBP 33-52
40	QKSHGRTQDENPVVHFFKNI	35	MBP 74-93
	DENPVVHFFKNIVT	36	MBP 84-97
	ENPVVHFFKNIVTPR	37	MBP 85-99
	HFFKNIVTPRTPP	38	MBP 90-102
	KGFKGVDAQGTLSK	39	MBP 139-152
45	VDAQGTLSKIFKLGGRDSRS	40	MBP 144-163
	LMQYIDANSKFIGITELKK	41	Tetanus Toxoid 828-846
	QYIKANSKFIGIT	42	Tetanus Toxoid 830-842
	FNNFTVSFWLRVPK	43	Tetanus Toxoid 947-960
	SFWLRVPKVSASHLE	44	Tetanus Toxoid 953-967
50	KFIIKRYTPNNEIDSF	45	Tetanus Toxoid 1174-1189
	GQIGNDPNRDIL	46	Tetanus Toxoid 1273-1284

TABLE 3: Tumor Antigens

Cancer	Associated Antigen
Melanoma	BAGE 2-10
Breast/Ovarian	c-ERB2 (Her2/neu)
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-1
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-2
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3A
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3C
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-4
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-6
Burkitt's lymphoma/Hodgkin's lymphoma	EBV
Burkitt's lymphoma/Hodgkin's lymphoma	EBV LMP2A
Melanoma	GAGE-1
Melanoma/Renal Cell Carcinoma/Glioma	gp75/TRP1
Cervical	HPV 16 E6
Cervical	HPV 16 E7
Cervical	HPV 18 E6
Cervical	HPV 18 E7
Melanoma	MAG
Melanoma	MAGE-1
Melanoma	MAGE-2
Melanoma	MAGE-3
Melanoma	MAGE-4b
Melanoma	MAGE-5
Melanoma	MAGE-6
Melanoma	MART-1/Melan-A
Pancreatic/Breast/Ovarian	MUC-1
Melanoma	MUM-1-B
Breast/Colorectal/Burkitt's lymphoma	p53
Melanoma	Pmel 17(gp100)
Prostate	PSA Prostate Specific Antigen
Melanoma/Breast/Glioma	Tyrosinase
Colorectal/Gastric/Pancreatic/Breast/Lung CEA	Carcinoembryonic Antigen
Lung	LRP Lung Resistance Protein
Lung/Colorectal/Prostate	hCG
Multiple	IGFR-1
Melanoma/Head/Neck/Renal Cell Carcinoma	NY-ESO-1
Lung/Ovarian/Bladder/Prostate	MAGE-A3
>85% of Human Cancers	hTERT
Breast/Glioma/Gastric/Ovarian/Squamous Cell	EGFR
Colorectal/Gastric/Ovarian/Osteosarcoma	CD55
Breast/Ovarian/Prostate/Pancreatic/Bladder	Her-2
Prostate	PAP

TABLE 4: Class I associated tumor and pathogen peptides

Peptide	SEQ ID NO:	Source Protein
AARAVFLAL	47	BAGE 2-10
YRPRPRRY	48	GAGE-1 9-16
EADPTGHSY	49	MAGE-1 161-169
SAYGEPRKL	50	MAGE-1 230-238
EVDPIGHLY	51	MAGE-3 161-169
FLWGPRALV	52	MAGE-3 271-279
GIGILTV	53	MART-1 29-35
ILTVILGV	54	MART-1 32-39
STAPPAHGV	55	MUC-1 9-17
EEKLIVVLF	56	MUM-1 261-269
MLLAVLYCL	57	TYROSINASE 1-9
SEIWRDIDF	58	TYROSINASE 192-200
AFLPWHRLF	59	TYROSINASE 206-214
YMNGTMSQV	60	TYROSINASE 369-376
KTWGQYWQV	61	PMEL 17 (GP100) 154-162
ITDQVPFSV	62	PMEL 17 (GP100) 209-217
YLEPGPTVA	63	PMEL 17 (GP100) 280-288
LLDGTATLRL	64	PMEL 17 (GP100) 476-485
ELNEALELEK	65	p53 343-351
STPPPGTRV	66	p53 149-157
LLPENNVLSPL	67	p53 25-35
LLGRNSFEV	68	p53 264-272
RMPEAAPPV	69	p53 65-73
KIFGSLAFL	70	HER-2/neu 369-377
IISAVVGIL	71	HER-2/neu 654-662
CLTSTVQLV	72	HER-2/neu 789-797
YLEDVRLV	73	HER-2/neu 835-842
VLVKSPNHV	74	HER-2/neu 851-859
RFRELVSEFSRM	75	HER-2/neu 968-979
LLRLSEPAEL	76	PSA 119-128
DLPTQEPAL	77	PSA 136-144
KLQCVD	78	PSA 166-171
VLVASRGRAV	79	PSA 36-45
VLVHPQWVL	80	PSA 49-57
DMSLLKNRFL	81	PSA 98-107
QWNSTAFHQ	82	HBV envelope 121-130
VLQAGFF	83	HBV envelope 177-184
LLLCLIFL	84	HBV envelope 250-257
LLDYQGML	85	HBV envelope 260-267
LLVPFV	86	HBV envelope 338-343
SILSPFMPLL	87	HBV envelope 370-379
PLLPIFFCL	88	HBV envelope 377-385
ILSTLPETTV	89	HBV core 529-538
FLPSDFFPSV	90	HBV core 47-56
KLHLYSHPI	91	HBV polymerase 489-498
ALMPLYACI	92	HBV polymerase 642-651
HLYSHPIIL	93	HBV polymerase 1076-1084
FLLSLGIHL	94	HBV polymerase 1147-1153
HLLVGSSGL	95	HBV polymerase 43-51
GLSRYVARL	96	HBV polymerase 455-463
LLAQFTSAI	97	HBV polymerase 527-535
YMDDVVLGA	98	HBV polymerase 551-559
GLYSSTVPV	99	HBV polymerase 61-69

	NLSWL	100	HBV polymerase 996-1000
	KLPQLCTEL	101	HPV 16 E6 18-26
	LQTTIHDII	102	HPV 16 E6 26-34
	FAFRDLCIV	103	HPV 16 E6 52-60
5	YMLDLQPET	104	HPV 16 E7 11-19
	TLHEYMLDL	105	HPV 16 E7 7-15
	LLMGTLGIV	106	HPV 16 E7 82-90
	TLGIVCPI	107	HPV 16 E7 86-93
	LLMGTLGIVCPI	108	HPV 16 E7 82-93
10	LLMGTLGIVCPICSQK	109	HPV 16 E7 82-97

In still other cases, the expression product includes an amino acid sequence essentially

identical to the sequence of an antigenic fragment of a protein naturally expressed by a virus,

e.g., a virus which chronically infects cells, such as human papilloma virus (HPV), human

immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis B virus (HBV), or

15   hepatitis C virus (HCV); a bacterium, such as mycobacteria or *Helicobacter pylori*; a fungus

such as *Candida, Aspergillus, Cryptococcus,* or *Histoplasmosis* species, or other eukaryotes,

such as a *Plasmodium* species. A representative list of such class I-binding fragments as well as

fragments of tumor antigens is included in Table 4. The MHC binding fragments (class I or

class II) can be encoded as part of a larger polytope compound of the type described in USSN

20   09/398,534 and USSN 60/154,665.

The nucleic acid in the microparticles described herein can be distributed either

throughout the microparticle, or in a small number of discrete regions within the microparticle.

Alternatively, the nucleic acid can be in the core of a hollow-core microparticle. The

microparticle preferably does not contain a cell (e.g., a bacterial cell), or a naturally occurring

25   genome of a cell.

The microparticles can also include a stabilizer. A stabilizer is a compound or

compounds that act to protect the nucleic acid (e.g., to keep it supercoiled or protect it from

degradation) at some point during the production and/or storage of the microparticles. Examples

of stabilizers include carbohydrates such as dextrose, sucrose, and trehalose; polyvinyl alcohol;

30   cyclodextrin; dextran; dextran sulfate; cationic compounds such as cationic peptides; buffering

agents such as TRIS, PBS, and MOPS; chelating agents such as EDTA and EGTA; DNase

inhibitors; pluronics, e.g., Pluronic F-68 (Sigma-Aldrich Co., St. Louis, MO); and lipids such as

CHAPS, PEG-DSPE, taurocholic acid, glycocholic acid, fatty acyl carnitine, N- lauryl sarcosine,

capric acid, vitamin D3, hexadecyltrimethylammonium bromide, QS21, purified saponin, or

35   polymyxin B.

Stabilizers can also be release modifiers such as carbohydrates, cationic compounds, pluronics, lipids (e.g., membrane destabilizing lipids), proteins, salts, peptides, surfactants, and small molecules. The stabilizer can remain associated with the DNA after the latter is released from the polymeric matrix. Moreover, although the term "stabilizer" is used in the singular

5   form, it should be understood to refer also to mixtures of two or more compounds (e.g., a carbohydrate and a buffer, or a carbohydrate, a buffer, and a lipid).

Among the advantages of the invention is that it provides an economical, aseptic, scalable procedure for producing a microparticle in amounts necessary for research, clinical, and other commercial uses. A microparticle produced using these procedures contains stable, active,

10   potent, structurally intact nucleic acid, e.g., as supercoiled DNA. The method also provides for efficient encapsulation of the nucleic acid in the microparticle and allows for efficient recovery of the microparticle.

Another advantage of the invention is that it affords microparticles that are substantially free of impurities, such as organic solvents used to prepare the microparticle. The microparticles

15   are suitable, therefore, for oral, rectal, vaginal, intranasal, intraarterial, intravenous, pulmonary, intramuscular, intradermal, transmucosal, intrathecal, intraperitoneal, transdermal, subdermal, or subcutaneous delivery.

Still another advantage of the invention is the control afforded over microparticle size, concurrent with scalable and reproducible large-scale production of microparticles.

20   Microparticle size can be important for a number of reasons, including delivery to a particular target site (e.g., lungs via inhalation), intravenous uptake by phagocytotic cells, and stability of the pharmaceutical suspension during storage and treatment. Moreover, the microparticles of the invention can be lyophilized, dried using a Sweco device (Emerson Electric Co., Florence, KY), or stored as a frozen liquid. Drying of microparticles can be accomplished as described in U.S.

25   6,080,429, incorporated herein by reference. Microparticles prepared in this manner are readily resuspended in a wide range of dispersing agents.

The microparticles can be administered to an animal (e.g., a mammal such as a human, non-human primate, horse, cow, pig, sheep, goat, dog, cat, mouse, rat, guinea pig, rabbit, hamster, or ferret). The microparticles can be provided suspended in a aqueous solution or any

30   other suitable formulation, and can be, for example, delivered orally, vaginally, rectally, nasally, buccally, or by inhalation, or injected or implanted (e.g., surgically) into the animal. They can

optionally be delivered in conjunction with a protein such as a cytokine or interferon, an antigen, a lipid, an adjuvant, or excipients that may be within or without the microparticle.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

5   belongs. Methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, although the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are

10   illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of a system for preparing nucleic acid-containing

15   microparticles according to the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The microparticles of the invention are formulated so as to maintain the integrity of the nucleic acid. For plasmid DNA, this means maximizing the percentage of plasmid molecules that are supercoiled and thus more stable than non-supercoiled (i.e., nicked or linear) plasmids

20   (Middaugh et al., *J. Pharm. Sci.* 87:130, 1998).

The nucleic acid can be RNA or DNA, or any known derivative of DNA (e.g., phosphorothiolate derivatives and the like typically used in antisense applications). In some embodiments, at least 50% (and preferably at least 70% or even 80%) of the nucleic acid is in the form of closed circles. The nucleic acid can be a linear or circular molecule, and can thus be,

25   e.g., a plasmid, or may include a viral genome, or part of a viral genome. When circular and double-stranded, it can be nicked, i.e., in an open circle, or super-coiled. In some embodiments the nucleic acids are plasmid molecules, at least 25% (and preferably at least 35%, 40%, 50%, 60%, 70%, 80%, or even 90% or more) of which are supercoiled.

The invention is not restricted to the manufacture of a microparticle having a particular type of nucleic acid. Any type of nucleic acid may be used. Examples include DNA which serves as the template for expression of an antisense RNA or a ribozyme, nucleic acids which encode a useful protein or peptide, and nucleic acids which themselves produce the desired

5   effect, such as antisense DNA or RNA, poly I:C, BCG DNA, oligonucleotides, and DNA having immunomodulating potential (e.g., CpG motifs).

The nucleic acid may encode a peptide or polypeptide that modulates, e.g., elicits, enhances, alters, or suppresses, a humoral or cell-mediated immune response. When modulation of cell-mediated immune responses is desired, the encoded product may be or include, for

10   example, an MHC class I- or MHC class II-binding peptide or polypeptide. Examples of nucleic acids encoding proteins or peptides that elicit immune responses are described in WO 95/23738; WO 97/17063; U.S. Patent No. 5,880,103; Jones et al., *Vaccine* 15:814, 1997; Chen et al., *J. Virology* 72:5757, 1998; Mathowitz et al., *Nature* 386:41, 1997; Jong et al., *J. Controlled Release* 47:123, 1997; and Hedley et al., *Nature Med.* 4:365, 1998, USSN 09/398,534, and

15   USSN 60/154,665.

The nucleic acid may alternatively elicit a non-specific immune response. One example of such a nucleic acid is poly I:C (i.e., a polyinosine:polycytosine double stranded nucleic acid), which induces an interferon response (see, e.g., EP A-0248 531). Another example is immunostimulatory CpG-oligodeoxynucleotides, which act as adjuvants for Th1 responses and

20   cytotoxic T cell responses to proteinaceous antigens (Sparwasser et al., *Eur. J. Immunol.* 28:2045, 1998). Another example is provided by nucleic acid encoding a polypeptide that regulates immune cells (e.g. T cells).

The nucleic acids can also include oligonucleotides which modulate gene expression, e.g., the antisense oligonucleotides described in Lewis et al., *J. Controlled Release* 37:173, 1995.

25   Alcohol precipitation of the nucleic acid prior to dissolution in the aqueous solution can improve encapsulation efficiency. Precipitation with ethanol can result in up to a 147% increase in incorporated DNA, and precipitation with isopropanol up to 170% (see, e.g., PCT US98/01499).

The nature of the aqueous solution can affect the yield of supercoiled or encapsulated

30   DNA. For example, addition of buffer solutions containing either tris(hydroxymethyl)-aminomethane (TRIS), ethylenediamine-tetraacetic acid (EDTA), or a combination of TRIS and

EDTA (TE), results in stabilization of supercoiled plasmid DNA, according to analysis by gel electrophoresis. Moreover, relatively high pH (e.g., 8.0 to 9.9) also has a stabilizing effect. Certain compounds, such as dextran sulfate, dextrose, dextran, CTAB, polyvinyl alcohol, trehalose, lipids, cyclodextrin, and sucrose, can enhance the stability and degree of supercoiling

5   of the DNA, either alone or in combination with the TE buffer. Stabilizers such as lipids and carbohydrates can also increase the overall amount of DNA encapsulated into the microparticles. Combinations of stabilizers can be used to increase the amount of encapsulated, supercoiled DNA. Stabilizers such as charged lipids (e.g., CTAB), cationic peptides, or dendrimers (*J. Controlled Release*, 39:357, 1996) can condense or precipitate the nucleic acid (e.g., DNA).

10   Moreover, stabilizers can have an effect on the physical nature of the particles formed during the encapsulation procedure. For example, the presence of sugars, lipids, or surfactants during the encapsulation procedure can generate porous or hollow particles with porous interior or exterior structures, allowing for a more rapid exit of a drug from the particle, rather than a slow controlled release over several days or months. The stabilizers can act at any time during the

15   preparation of the microparticles (e.g., during encapsulation or lyophilization, or both) or during the degradation of the polymer *in vivo*.

The microparticles of the invention are generally formulated in one of two ways: (1) to maximize delivery into the patient's phagocytic cells, or (2) to form a deposit in the tissues of the patient, from which the nucleic acid is released gradually over time; upon release from the

20   deposit, the nucleic acid is taken up by neighboring cells (including antigen presenting cells, or APCs). In both cases, maintaining the integrity of the DNA is a priority. For plasmid DNA, this means maximizing the percentage of plasmid molecules that are supercoiled and which may be capable of more efficient transfection and transcription than non-supercoiled (i.e., nicked or linear) plasmids. Maximizing the percentage of supercoiled plasmid molecules may also

25   increase the stability of the DNA in the cell or microparticle, as well as the shelf life of the nucleic acid-containing microparticles.

Means for protecting the integrity of the nucleic acid include minimizing the shearing forces to which the nucleic acid is necessarily exposed in the process of microparticle formation, limiting sonication, homogenization, microfluidization, or other mixing times during preparation,

30   controlling lyophilization, drying, or hardening, adding buffers or other stabilizers during microparticle preparation, and limiting the time that nucleic acid is exposed to high temperatures

(e.g., limiting exposure to temperatures above about 39°C to less than about an hour). For example, it is desirable to achieve a balance between homogenization time and intensity which minimizes shear yet produces the desired size of microparticles with an acceptably high encapsulation efficiency (i.e. an encapsulation efficiency of $\geq 25\%$ of the nucleic acid). These techniques are discussed below.

The microparticles of the invention can be used in the manufacture of a medicament for the treatment of, for example, cancer, infectious disease, any of the autoimmune diseases listed in Table 1, or any other condition treatable with a particular defined nucleic acid.

Phagocytosis of microparticles by macrophages, dendritic cells, and other APCs is an effective means for introducing the nucleic acid into these cells. Phagocytosis by these cells can be increased by maintaining a particle size below about 20 μm, preferably below about 11 μm, and most preferably below about 5 μm. The type of polymer used in the microparticle can also affect the efficiency of uptake by phagocytic cells, as discussed below.

The microparticles can be delivered directly into the bloodstream (i.e., by intravenous or intraarterial injection or infusion) where uptake by the phagocytic cells of the reticuloendothelial system (RES) is desired. Alternatively, the microparticles can be delivered orally (e.g., to Peyers patches or mesenteric lymph nodes, mucosally, nasally, buccally, vaginally, rectally or intralesionally). The microparticles can also be delivered via subcutaneous injection, to facilitate take-up by the phagocytic cells of the draining lymph nodes. Alternatively, the microparticles can be introduced intradermally (i.e., to the APCs of the skin, such as dendritic cells and Langerhans cells) or intramuscularly. Finally, the microparticles can be introduced into the lung (e.g., by inhalation of powdered microparticles or of a nebulized or aerosolized solution or suspension containing the microparticles), where the particles are picked up by the alveolar macrophages.

Once a phagocytic cell phagocytoses the microparticle, the nucleic acid is released into the interior of the cell. Upon release, it can perform its intended function: for example, expression by normal cellular transcription/translation machinery (for an expression vector), or alteration of cellular processes (for antisense or ribozyme molecules, or CpG or poly-I:C containing nucleic acids).

Because these microparticles are passively targeted to macrophages and other types of professional APC and phagocytic cells, they represent a means for modulating immune function.

Macrophages and dendritic cells serve as professional APCs, expressing both MHC class I and class II molecules. In addition, the mitogenic effect of DNA can be used to stimulate non-specific immune responses mediated by B, T, and NK cells; macrophages; and other cells.

5   Delivery, via microparticles, of an expression vector encoding a foreign antigen which binds to an MHC class I or class II molecule will induce a host T cell response against the antigen, thereby conferring host immunity.

Where the expression vector encodes a blocking peptide (See, e.g., WO 94/04171) that binds to an MHC class II molecule involved in autoimmunity, presentation of the autoimmune disease-associated self peptide by the class I molecule is prevented, and the symptoms of the
10   autoimmune disease alleviated.

In another example, an MHC binding peptide that is identical or almost identical to an autoimmunity-inducing peptide can affect T cell function by tolerizing or anergizing the T cell. Alternatively, the peptide could be designed to modulate T cell function by altering cytokine secretion profiles following recognition of the MHC/peptide complex. Peptides recognized by
15   T cells can induce secretion of cytokines that cause B cells to produce antibodies of a particular class, induce inflammation, and further promote host T cell responses.

Induction of immune responses, e.g., specific antibody responses to peptides or proteins, can require several factors. It is this multifactorial nature that provides impetus for attempts to manipulate immune related cells on multiple fronts, using the microparticles of the invention.
20   For example, microparticles can be prepared which carry both DNA and peptides, polypeptides, and/or adjuvants within each microparticle; alternatively, separate batches of microparticles can be prepared each of which carries only one of these substances, and then mixed. These dual-function microparticles are discussed below.

CTL Responses

25   Class I molecules present antigenic peptides to immature T cells. To fully activate T cells, factors other than the antigenic peptide are required. Non-specific proteins such as interleukin-2 (IL-2), IL-12, and gamma interferon (γ-IFN) promote CTL responses and can be provided together with DNA encoding polypeptides which include CTL epitopes. Alternatively, proteins which bear helper T (Th) determinants can be included with DNA encoding the CTL

epitope or epitopes. T helper epitopes promote secretion of cytokines from T helper cells and play a role in the differentiation of nascent T cells into CTLs.

Alternatively, peptides, proteins, nucleic acids, or adjuvants which promote migration, differentiation, or proliferation of lymphocytes and macrophages to a particular area could be

5 included in microparticles, along with appropriate DNA molecules. Uptake of the DNA is enhanced as a result, because release of the protein would cause an influx of phagocytic cells and T cells as the microparticle degrades. The macrophages would phagocytose the remaining microparticles and act as APC, and the T cells would become effector cells.

Antibody Responses

10 Elimination of certain infectious agents from the host may require both antibody and CTL responses. For example, when influenza virus enters a host, antibodies can often prevent it from infecting host cells. However, if cells are infected, then a CTL response is required to eliminate the infected cells and to prevent the continued production of virus within the host.

In general, antibody responses are directed against conformational determinants and thus

15 require the presence of a protein or a protein fragment containing such a determinant. In contrast, T cell epitopes are linear determinants, typically just 7-25 residues in length. Thus, when there is a need to induce both a CTL and an antibody response, the microparticles can include both an antigenic protein and the DNA encoding a T cell epitope. Slow release of the protein from microparticles outside of cells would lead to B cell recognition and subsequent

20 secretion of antibody, while phagocytosis of the microparticles would cause APCs (1) to express the DNA of interest, thereby generating a T cell response; and (2) to digest the protein released from the microparticles, thereby generating peptides which are subsequently presented by class I or II molecules. Presentation by class I or II molecules promotes both antibody and CTL responses, since T helper cells activated by the class II/peptide complexes would secrete non-

25 specific cytokines.

Microparticles for Implantation

A second microparticle formulation of the invention is intended not to be taken up directly by cells, but rather to serve primarily as a slow-release reservoir of nucleic acid that is taken up by cells only upon release from the microparticle. Release may occur, for example, by

30 degradation, erosion, or diffusion. The nucleic acid can be complexed to a stabilizer, e.g., to

-18-

maintain the integrity of the nucleic acid during the slow-release process. The polymeric particles in this embodiment should therefore be large enough to minimize the extent of phagocytosis (i.e., larger than 5 μm and preferably larger than 20 μm). Such particles are produced by the methods described for making the smaller particles, but with less vigorous

5 mixing. That is to say, a lower homogenization speed can be used to obtain particles having a diameter around 100 μm rather than 5 μm. The time of mixing, the viscosity of the first emulsion, and the concentration of polymer in the first solution can also be altered to affect particle dimension.

The larger microparticles can be formulated as a suspension, a powder, or an implantable

10 solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intrapulmonary); orally, e.g. in the form of a tablet; intranasally or by implantation. These particles are useful for delivery of any expression vector or other nucleic acid for which slow release over a relatively long term is desired: e.g., an antisense molecule, a gene replacement therapeutic, a means of delivering cytokine-based, antigen-based, or hormone-

15 based therapeutic, or an immunosuppressive agent. The rate of degradation, and consequently of release, varies with the polymeric formulation. This parameter can be used to control immune function. For example, one might want a relatively slow release for delivery of IL-4 or IL-10, and a relatively rapid release for delivery of IL-2 or γ-IFN.

Composition of Polymeric Particles

20 Polymeric material is obtained from commercial sources or can be prepared by known methods. For example, polymers of lactic and glycolic acid can be generated as described in US Patent No. 4,293,539 or purchased from a commercial source such as Aldrich Chemicals, Birmingham Polymers, or Boehringer Ingelheim. One suitable polymer is poly-lactic-*co*- glycolic acid) (PLGA), with a lactic/glycolic acid weight ratio of about 1:2 to about 4:1

25 (e.g., 50:50, 65:35, or 75:25).

Alternatively, or in addition, the polymeric matrix can include any one or more of polylactide, polyglycolide, polyanhydride, polyorthoester, polycaprolactone, polyphosphazene, polypeptide, polyester, or naturally occurring polymers such as alginate, chitosan, and gelatin.

Preferred controlled release substances which are useful in the formulations of the

30 invention include the polyanhydrides, co-polymers of lactic acid and glycolic acid wherein the

weight ratio of lactic acid to glycolic acid is no more than 4:1, and polyorthoesters containing a degradation-enhancing catalyst, such as an anhydride, e.g., 1% maleic anhydride. Since polylactic acid can take at least one year to degrade *in vivo*, this polymer should be utilized by itself only in circumstances where extended degradation is desirable.

5   In some cases, the polymeric matrix also includes a targeting molecule such as a ligand, receptor, or antibody, to increase the specificity of the microparticle for a given cell type or tissue type.

Preparation of Microparticles:

Microparticles are prepared by making a first emulsion (i.e., via a batch process or a
10   continuous flow process) from an aqueous solution containing a nucleic acid and a solution containing a polymeric material dissolved in an organic solvent (e.g., dichloromethane, phenol, chloroform, or ethyl acetate), and then combining the first emulsion with a surfactant-containing second aqueous solution (e.g., a solution of poly(vinyl alcohol), PVA; oleic acid; TWEEN®; SPAN®; poly(vinylpyrrolidone), PVP; other surface-reactive agents) to produce a second
15   emulsion from which the microparticles are isolated. The microparticles are then purified and optionally concentrated, for example, by diafiltration, or using a Sweco™ device (Emerson Electric Co., Florence, KY).

Diafiltration is a process by which particles and relatively high molecular weight solutes can be separated from relatively low molecular weight solutes, where both are present in the
20   same suspension. The suspension is diluted with solvent (e.g., water) and passed through a hollow fiber membrane, washing away the relatively low molecular weight solutes from the retentate. In the present methods, diafiltration can be used to remove excess surfactants (e.g., PVA), stabilizers (e.g., sucrose), and buffers (e.g., TE) from the microparticles.

Sweco devices such as the PharmASep Filter-Dryer™ (Emerson Electric Co., Florence,
25   KY) can be used in place of diafiltration. A liquid slurry of the microparticles is pumped into the Sweco device for purification. As the liquid passes through the device, the solid microparticles are restrained by a vibrating fine mesh screen. The microparticles can be washed with water-for-injection to remove soluble contaminants (e.g., PVA, sucrose). Optionally, the microparticles can also be coated with an excipient in the device (i.e., by washing with a solution of the
30   excipient). Examples of excipients are PEG, carboxymethylcellulose, sorbitol, TWEEN, and

mannitol. A flow of sterile air or nitrogen is then passed through the device to dry the microparticles. The dried microparticles, in the form of a powder, are discharged through an exit port into a sterile receiving vessel. A powder augur is then used to carry the powder up a tube at a measured rate and into individual vials. Alternatively, microparticles can be washed to remove

5   solvent, concentrated using a vibrating or non-vibrating stainless steel mesh screen device, and then dried (e.g., in a lyophilizer).

In the processes of the invention, the first emulsion and the surfactant-containing solution are pumped into a mixing chamber, including, for example a microfluidizer (e.g., as available from Microfluidics, Newton, MA), a French press (e.g., as available from Microfluidics or

10   Gaulin-APV), a mechanical homogenizer (e.g., as available from Silverson, VirTis, or Ika Works), or an ultrasonic emulsifier (e.g., as available from Branson Ultrasonics Corporation or VirTis). In the mixing chamber, the first emulsion and the surfactant-containing solution are blended to form a second emulsion. The surfactant-containing solution can optionally include a stabilizer, such as a nucleic acid release modulator, a buffer, a carbohydrate or a lipid. It is

15   important that the second aqueous solution have an osmolarity, pH, and buffering capacity essentially identical to that of the first aqueous solution to minimize loss of the nucleic acid into the aqueous phase of the second emulsion.

As the first emulsion and surfactant-containing solution are pumped into the mixing chamber, the second emulsion can be continuously pumped out of the mixing chamber into a

20   solvent removal device. The solvent removal device may be any vessel (e.g., a closed vessel) that can be sterilized. Examples of suitable solvent removal devices include pharmaceutical pressure vessels (e.g., as available from Eagle Stainless Container, Alloy Products Corporation, DCI Inc., Walker Stainless Equipment), bioreactors/fermenters (e.g., as available from Applikon, New Brunswick, Chemap, B. Braun, and LH), and hollow-fiber membrane devices. In the

25   solvent removal device, the organic solvent is removed (e.g., by evaporation with heating (e.g., to 30-55°C) or an applied vacuum; by extraction; by addition of an alcohol such as isopropanol or ethanol; by dilution with water; or using hollow fiber membranes.

During removal of the organic solvent, microparticles containing nucleic acid encapsulated in a polymeric matrix or shell precipitate from the emulsion, forming a suspension.

30   Following removal of the organic solvent, the microparticle-containing suspension can be continuously removed from the solvent removal device into a filtration or wash reservoir. The

contents of the reservoir are diluted with an aqueous wash solution (e.g., water) and pumped into a filtration apparatus (e.g., a diafiltration apparatus). Aqueous solution is removed from the apparatus, leaving the microparticles behind. The removed solution includes, for example, any excess surfactant that is not incorporated into the microparticles. The suspension of

5   microparticles can be concentrated by removing the aqueous solution from the filtration apparatus at a higher rate than the wash solution is pumped in, or by ceasing the inflow of the wash solution altogether. The final, concentrated suspension can contain excipients such as mannitol, PEG, carboxymethylcellulose, sorbitol, and TWEEN. The suspension can then be aliquotted into single dose vials. Freezing and lyophilizing the filtered microparticle suspension

10   in a single dose vial results in an easily stored dry preparation. Alternatively, the solution containing the particles can be pumped onto a fine mesh sieve such as a Sweco device for washing and drying. The sieve would allow the aqueous solution to pass through while retaining the particles. Particles washed on a Sweco unit would be dried by passing sterile air over the particles. These particles would be removed from the Sweco unit into a holding vessel. From

15   here the particles could be aliquotted into single dose vials by way of a rotating screw that deposits a predetermined amount of particles into each vial. The vials would then be sealed for storage. Particles may also be retained in a Sweco device for washing, and then dried in a lyophilizer before being aliquotted into single dose vials.

It is important to generate microparticles with very little residual organic solvent, so that

20   the particles are suitable for use in animals. The amount of residual organic solvent in the dried or lyophilized particles is preferably below 200 ppm, and more preferably below 50 ppm.

It is also important that the particles be prepared in an aseptic fashion (e.g., as defined in U.S. Pharmacopeia USP24<71>) if they are to be used in the treatment of disease in animals (e.g., humans). The processes described herein have been designed such that sterility can be

25   maintained throughout and the resulting product is essentially free of biological contaminants. Techniques for sterilizing machinery and filtering solutions aseptically are known by those skilled in the art of pharmaceutical manufacturing (e.g., good manufacturing practice ("GMP") manufacturers of biological products), and are employed in the present processes.

Larger particles, such as those used for implantation, can be obtained by using less

30   vigorous emulsification conditions when making the second emulsion, by altering the concentration of the polymer, altering the viscosity of the emulsion, altering the particle size of

the first emulsion (e.g., larger particles can be made by decreasing the pressure used while creating the first emulsion in a microfluidizer), or homogenizing with, for example, the Silverson homogenizer set at 5000 rpm for about 12 seconds.

5   The washed, or washed and lyophilized, or washed and dried microparticles can be suspended in an excipient without negatively affecting the amount of supercoiled plasmid DNA within the microparticles. Excipients such as saline, carbohydrates, detergents and other surfactants, polymers, buffers, and lipids are often used in drug formulation, and here provide for efficient microparticle resuspension, act to prevent settling, and/or increase *in vivo* dispersion. According to analysis by gel electrophoresis, excipients such as TWEEN 80, mannitol, sorbitol,

10   and carboxymethylcellulose do not have a deleterious effect on nucleic acid stability or supercoiling, when included prior to or after lyophilization.

Characterization of Microparticles

The size distribution of the microparticles prepared by the above method can be determined with a COULTER™ counter. This instrument provides a size distribution profile and

15   statistical analysis of the particles. Alternatively, the average size of the particles can be determined by visualization under a microscope fitted with a sizing slide or eyepiece.

If desired, the nucleic acid can be extracted from the microparticles for analysis by the following procedure. Microparticles are dissolved in an organic solvent such as chloroform or methylene chloride in the presence of an aqueous solution. The polymer stays in the organic

20   phase, while the nucleic acid goes to the aqueous phase. The interface between the phases can be made more distinct by centrifugation. Isolation of the aqueous phase allows recovery of the nucleic acid. The nucleic acid is retrieved from the aqueous phase by precipitation with salt and ethanol in accordance with standard methods. To test for degradation, the extracted nucleic acid can be analyzed by HPLC, capillary gel electrophoresis, or agarose gel electrophoresis.

25   The amount of residual organic solvent in the particles can be determined by methods known in the art, such as, for example, gas chromatography or gas chromatography coupled to mass spectrometry. More specifically, microparticles were produced by the procedure detailed in Example 2 and were tested for residual solvent as follows:

Four vials representing various stages of the vial fill were sequestered for analysis.

30   Approximately 15 mg of lyophile was removed from each of the four vials and combined with

gentle mixing (using a hand-held spatula) to ensure homogeneity of the test article. The pooled lyophile was then resuspended in tetrahydrofuran with an internal standard (1,2-dichloro-propane), to adjust for variations in injection volume from injection to injection. Each test article was subjected to duplicate injections into a gas chromatograph outfitted with a Supelco™

5   SPB-5 30m X 0.53mm column and an electron capture detector. Peak areas of dichloromethane DCM and injection control were determined (both peaks achieve baseline resolution). The DCM/1,2-dichloropropane ratio was determined for the test article and was compared to the corresponding ratio from a standard prepared to a known concentration. This comparison yielded the concentration of DCM in the test article. The test method is quantitative in the 50 to

10   100 ppm DCM concentration range, with a limit of quantitation of approximately 30 ppm and a limit of detection of approximately 20 ppm. The procedure was applied to two preparations of microparticles. One preparation was found to have a residual DCM level at around the limit of detection (i.e., about 20 ppm). The other had a residual DCM level of 79 ppm.

Non-GMP samples were analyzed in a similar manner. Lyophilized test article was

15   resuspended in THF with 1,2-dichloropropane and subjected to GC analysis as previously described. The liquid test article was injected directly into the GC without benefit of sample preparation. A DCM standard curve was generated for each sample set and DCM concentrations of test articles are extrapolated from the standard curve.

In Vivo Delivery of Microparticles

20   Microparticles containing nucleic acid can be resuspended in saline, buffered salt solution, tissue culture medium, carbohydrate- or lipid-containing solution, or other physiologically acceptable carrier. They can be injected into an animal (e.g., a mammal such as a human) intramuscularly, intravenously, intraarterially, intradermally, intrathecally, intraperitoneally, or subcutaneously, or they can be introduced into the gastrointestinal tract or

25   the respiratory tract, e.g., by inhalation of a suspension or powder containing the microparticles, or swallowing a tablet or suspension containing the microparticles. Alternatively, the microparticles can be introduced into a mucosal site such as the vagina, nose, or rectum. Expression of the nucleic acid is monitored by an appropriate method. For example, expression of a nucleic acid encoding an immunogenic protein of interest is assayed by RT-PCR or by

30   looking for an antibody or T cell response to the protein.

Antibody responses can be measured by testing serum in an ELISA assay. In this assay, the protein of interest is coated onto a 96 well plate and serial dilutions of serum from the test subject are pipetted into each well. A secondary, enzyme-linked antibody, such as anti-human, horseradish peroxidase-linked antibody, is then added to the wells. If antibodies to the protein of

5   interest are present in the test subject's serum, they will bind to the protein fixed on the plate, and will in turn be bound by the secondary antibody. A substrate for the enzyme is added to the mixture and a colorimetric change is quantitated in an ELISA plate reader. A positive serum response indicates that the immunogenic protein encoded by the microparticle's DNA was expressed in the test subject, and stimulated an antibody response. Alternatively, an ELISA spot

10   assay can be employed.

T cell proliferation in response to a protein following intracellular delivery of microparticles containing nucleic acid encoding the protein is measured by assaying the T cells present in the spleen, lymph nodes, or peripheral blood lymphocytes of a test animal. The T cells obtained from such a source are incubated with syngeneic APCs in the presence of the protein or

15   peptide of interest. Proliferation of T cells is monitored by uptake of ^{3}H-thymidine, according to standard methods. The amount of radioactivity incorporated into the cells is directly related to the intensity of the proliferative response induced in the test subject by expression of the microparticle-delivered nucleic acid. A positive response indicates that the microparticle containing DNA encoding the protein or peptide was taken up and expressed by APCs *in vivo*.

20   The generation of cytotoxic T cells can be demonstrated·in a standard ^{51}Cr release assay (see, e.g., PCT application WO99/18995, USSN 09/398,534, or USSN 60/154,665). In these assays, spleen cells or peripheral blood lymphocytes obtained from the test subject are cultured in the presence of syngeneic APCs (e.g., dendritic cells) and either the protein of interest or an epitope derived from this protein. After a stimulation cycle (e.g., 4-10 days), the effector

25   cytotoxic T cells are mixed with ^{51}Cr-labeled target cells expressing an epitope derived from the protein of interest. Effector cells can be stimulated in vitro 1-3 times. If the test subject raised a cytotoxic T cell response to the protein or peptide encoded by the nucleic acid contained within the microparticle, the cytotoxic T cells will lyse the targets. Lysed targets will release the radioactive ^{51}Cr into the medium. Aliquots of the medium are assayed for radioactivity in a

30   scintillation counter. Assays, such as ELISA or FACS, can also be used to measure cytokine

profiles of responding T cells (see, e.g., PCT application WO99/18995, USSN 09/398,534, or USSN 60/154,665).

Lipid-Containing Microparticles

The microparticles described herein can also include one or more types of lipids as

5 stabilizing compounds. The inclusion of a lipid in a microparticle can increase the stability of the nucleic acid in the microparticle, e.g., by maintaining a covalently closed double-stranded DNA molecule in a supercoiled state. The presence of a lipid in the particle can modulate, i.e., increase or decrease, the rate at which a drug or nucleic acid is released from the microparticle.

Addition of a lipid to the microparticle can in certain cases increase the efficiency of

10 encapsulation of the nucleic acid or increase the loading of the nucleic acid within microparticles. For example, the encapsulation efficiency may be improved because the presence of the lipid reduces the surface tension between the inner aqueous phase and the organic phase. Reduction of the surface tension is thought to create an environment more favorable for the nucleic acid, and therefore to increase its retention within the microparticle.

15 A reduction in surface tension also allows for the primary emulsion to be formed with less manipulation, which minimizes shearing of the nucleic acid and increases supercoiling. It is also possible that the presence of lipid in the microparticle may enhance the stability of the microparticle/nucleic acid formulation, and may increase the hydrophobic nature of the microparticles, thereby increasing uptake by phagocytic cells.

20 The lipids can be cationic, anionic, or zwitterionic, or may carry no charged groups, such as nonpolar glycerides. They can be, for example, fatty acids, eicosanoids, glycerophospholipids, triacetylglycerols, waxes, sphingolipids, steroids (e.g., cholesterol, CHAPS, bile acids, hormones, cardiac aglycones), lipid vitamins, or terpenes. The lipids preferably are not present as liposomes that encapsulate (i.e., surround) the microparticles. The

25 lipids may optionally form micelles.

Examples of lipids that can be used in the microparticles include acids (e.g., carboxylic acids, including fatty acids such as capric acid), bases (such as amines), zwitterionic lipids (e.g., CHAPS), phospholipids such as phosphatidylethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, phosphatidylcholine, or phosphatidic acid, each containing one or

30 more of the following groups: propionyl (propanoyl), butyryl (butanoyl), valeryl (pentanoyl), caproyl (hexanoyl), caprylyl (heptanoyl), capryl (decanoyl), undecanoyl, lauryl (dodecanoyl),

tridecanoyl, myristyl (tetradecanoyl), pentadecanoyl, palmityl (hexadecanoyl), phytanoyl (3,7,11,15-tetramethylhexadecanoyl), heptadecanoyl, stearyl (octadecanoyl), bromostearyl, nonadecanoyl, arachidoyl (eicosanoyl), heneicosanoyl, behenyl (docosanoyl), tricosanoyl, lignoceryl (tetracosanoyl), myristoleoyl (9-*cis*-tetradecanoyl), myristelaidoyl (9-*trans*-

5   tetradecanoyl), palmitoleyl (9-*cis*-hexadecanoyl), palmitelaidyl (9-*trans*-hexadecenoyl), petroselinoyl (6-*cis*-octadecenoyl), oleoyl (9-*cis*-octadecenoyl), elaidoyl (9-*trans*-octadecenoyl), linoleoyl (9-*cis*-12-*cis*-octadecadienoyl), linolenoyl (9-*cis*-12-*cis*-15-*cis*-octadecatrienoyl), eicosenoyl (11-*cis*-eicosenoic), arachidonyl (5,8,11,14-(all-*cis*)-eicosatetraenoic), erucoyl (13-*cis*-docosenoic), and nervonoyl (15-*cis*-tetracosenoic).

10   Other suitable lipids include the cetyltrimethyl ammonium ion, which is available as CTAB, PEG-DSPE, and those that contain steroid structures such as cholesterol, CHAPS, and certain vitamins and hormones. Saponin-derived lipids (e.g., QS21) may also be used.

Mixtures of lipids can be used to make a lipid-containing microparticle. Suitable commercially available lipid preparations include lecithin, OVOTHIN 160™, and EPIKURON

15   135F™ lipid suspensions, all of which are available from Lucas Meyer, Inc., Decatur, IL.

The lipid may also be isolated from an organism, e.g., a mycobacterium. The lipid is preferably a CD1-restricted lipid, such as the lipids described in Pamer, *Trend Microbiol.* 7:13, 1999; Braud, *Curr Opin. Immunol.* 11:100, 1999; Jackman, *Crit. Rev. Immunol.* 19:49, 1999; and Prigozy, *Trends Microbiol.* 6:454, 1998.

20   Instead of, or in addition to, incorporating lipids into the microparticles, the microparticles can be suspended in a lipid (or lipid suspension) to improve dispersion, delivery following injection, or to keep them in a suspended state.

The lipid-containing microparticles may also include additional stabilizers, as described above. The inclusion of a lipid in a microparticle along with a stabilizer such as sucrose can

25   provide a synergistic increase in the release of nucleic acids within the microparticle.

As described above, lipid-containing microparticles can be prepared by adding a lipid to the organic solvent containing the polymer, to the aqueous solution containing the DNA solution, or to the aqueous solution containing the surfactant. The solubility properties of a particular lipid in an organic or aqueous solvent will determine which solvent is used.

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Some lipids or lipid suspensions can be added to either the organic solvent or the aqueous solution. Microparticles may in addition be resuspended in a lipid-containing solution to facilitate resuspension and dispersion of the microparticles.

In addition to the lipid-containing microparticles described herein, microparticles may also be made by incorporating into the polymer solution (i.e., along with PLGA) other macromolecules such as chitin, gelatin, or alginate, or various combinations of these macromolecules and lipids. Microparticles made with these other macromolecules may in addition include the above-described stabilizing agents.

The method is illustrated by the following non-limiting examples.

EXAMPLES

EXAMPLE 1: Preparation of DNA

500 ml bacterial cultures containing plasmid DNA were poured into one liter centrifuge bottles. The cultures were centrifuged at 4000 rpm at 20°C for 20 minutes. The medium was removed from the pelleted bacteria. The bacterial pellet was completely resuspended in 50 ml buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase), leaving no clumps. 50 ml of buffer P2 (200 mM NaOH, 1% SDS) was added with gentle swirling, and the suspensions were incubated at room temperature for five minutes to lyse the cells. 50 ml of buffer P3 (3.0 M potassium acetate, pH 5.5, chilled to 4°C) was added with immediate, gentle mixing. The suspensions were incubated on ice for 30 minutes, then centrifuged at 4000 rpm at 4°C for 30 minutes.

A folded, round filter was wetted with water. When the centrifugation was complete, the supernatant was immediately poured through the filter. The filtered supernatant was collected in a clean 250 ml centrifuge bottle.

15 ml of Qiagen ER™ buffer was added to the filtered lysate, mixing by inverting the bottle 10 times. The lysate was incubated on ice for 30 minutes.

A Qiagen-tip 2500™ column was equilibrated by applying 35 ml Qiagen QBT™ buffer (750 mM sodium chloride; 50 mM MOPS, pH 7.0; 15% isopropanol; and 0.15% Triton X-100). The column was allowed to empty by gravity flow. The incubated lysate was applied to the column and allowed to enter by gravity flow. The column was washed with 4 x 50 ml Qiagen Endofree QC™ buffer (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The DNA was eluted from the column with 35 ml of QN™ buffer (1.6 M NaCl; 50 mM MOPS, pH 7.0;

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15% isopropanol) into a 50 ml polypropylene screwcap centrifuge tube. The DNA suspension was split into two tubes by pouring approximately 17.5 ml of the suspension into a second 50 ml screwcap tube.

Using a sterile 10 ml pipette, 12.25 ml isopropanol was added to each tube. The tubes
5   were closed tightly and thoroughly mixed. The contents of each tube were poured into 30 ml Corex™ (VWR) centrifuge tubes. Each Corex tube was covered with PARAFILM®. The tubes were centrifuged at 11,000 rpm at 4°C for 30 minutes.

The supernatant was aspirated from each tube and the pellet was washed with 2 ml 70% ethanol. The ethanol was aspirated off. The pellet was air dried for 10 minutes, then
10   resuspended in 0.5-1.0 ml water and transferred to a sterile 1.5 ml microfuge tube.

EXAMPLE 2: Preparation of Microparticles

Referring to Fig. 1, a bottle 5 containing 50 ml of dichloromethane (DCM) into which had been dissolved 6 g of poly-lactic-*co*-glycolic acid ("PLGA/DCM"), was placed in an ice tub. The bottle 5 was fitted with a Silverson SL2T™ Homogenizer 7.

15   10.8 ml of a DNA/sucrose/TE solution (i.e., 72 mg DNA, 300 mM sucrose, and 10 mM TE) was injected into the PLGA/DCM solution using a syringe. Homogenization at 10,000 rpm was begun concurrently with the injection of the DNA solution, and continued for 15 minutes.

An additional 100 ml of DCM was then injected into the resulting emulsion with a syringe over a period of not more than 20 seconds. After the injection of the 100 ml DCM, the
20   homogenization was allowed to continue for an additional 30 seconds, and homogenizer 7 was then stopped. Bottle 5 containing the first emulsion was then removed from the ice tub and attached to conduit 14. Conduit 14 was routed through pump 16 to homogenizing chamber 23 fitted with a Silverson L4RT™ Homogenizer 24.

Microparticles were prepared by homogenizing the first emulsion with a surfactant. The
25   mixing chamber 23 was in line with a reservoir 18 containing 1% polyvinyl alcohol (PVA)/10.37% sucrose solution. The reservoir 18 was connected via conduit 20 through pump 22 to the mixing chamber 23.

The PVA/sucrose solution was drawn from reservoir 18 through pump 22, set at a flow rate of 1 l/min. Immediately after beginning the flow of the PVA/sucrose solution, the
30   homogenizer 24 was set to 6,000 rpm. A second emulsion was then formed by drawing the first

emulsion from bottle 5 into chamber 23 by setting pump 16 to a flow rate of 36 ml/min. After homogenization, the second emulsion exited through conduit 25 and entered a BIOFLOW 2000® bioreactor chamber 28 (New Brunswick Scientific, Edison, NJ) containing a stirrer operating at 50 rpm. Conduit 25 was fitted with clamp 26 to regulate flow of the second

5   emulsion between homogenizer 24 and bioreactor chamber 28.

       Bioreactor chamber 28 also featured inlet 32 for delivering nitrogen, pressure relief valve 34, and outlet conduit 36 to which was attached clamp 38. Conduit 36 was in communication with "Y"-connector 40, which diverted flow from conduit 36 into conduit 42. Fluid flow through conduit 42 was regulated by clamp 44. Alternatively, fluids could be introduced into conduit 48

10   via conduits 41 and 42 by closing clamp 38 and opening clamps 43 and 44.

       Five liters of the PVA/sucrose solution from reservoir 18 and 150 ml of the first emulsion from bottle 5 were pumped into homogenizer 24, and then into bioreactor chamber 28, over a five minute period. After the solutions had been pumped through the homogenizer 24, the stirring speed in the bioreactor chamber 28 was increased to 375 rpm for 30 minutes.

15   The temperature of the bioreactor chamber 28 was set to 22°C and monitored every 5 minutes. After 30 minutes, the temperature set point was increased to 37°C, and the temperature was again monitored every 5 minutes. When the temperature had reached 37°C, the stirring speed was maintained at 375 rpm and stirring was continued for 1.5 hours, after which time the stirring speed was adjusted to 150 rpm and the temperature set point was decreased to

20   15°C.

       Washing of the microparticles was initiated by introducing 500 ml of water (4°C) through conduits 41 and 42 (i.e., with clamp 38 closed), pump 46, and conduit 48, to an AG Technologies hollow fiber reservoir 50. The flow rate through hollow fiber reservoir 50 was initially set at a rate of 11 l/min via pump 57.

25   After addition of the 500 ml water to hollow fiber reservoir 50, clamp 43 was closed, and clamp 38 released. Pump 46 was then used to transfer solution from bioreactor chamber 28 to hollow fiber reservoir 50 at a flow rate of 80 ml/min.

       Hollow fiber reservoir 50 was connected to a pressure release vent 52, which had a filter 54, and to conduits 55 and 56. Fluid was circulated from the hollow fiber reservoir 50 through

30   conduit 56 via pump 57 to serially stacked hollow fiber cartridges 59, each having polysulfone porous membranes with 0.2 μm pores. Fluid entering hollow fiber cartridges 59 from conduit 56

passed into the interior region of the hollow fibers. This region, in turn, was in communication with conduit 64. The size of the hollow fiber membrane's pores was sufficiently small so that the microparticles could not pass through the membranes. In contrast, reagents and byproducts of the manufacturing process, e.g., the PVA and sucrose, did pass through the membranes. This

5 permeate was removed through conduit 62 by pump 66 using a flow rate of 40 ml/min. The total flow rate was thus 80 ml/min for the two chambers.

Conduit 56 from hollow fiber reservoir 50 was also connected to clamp 70, which regulated flow of liquid into a 600 ml glass beaker 72. As explained in more detail below, release of clamp 70 allowed for delivery of microparticles from the hollow fiber reservoir 50 to

10 beaker 72.

With clamp 70 closed, fluid was directed from hollow fiber reservoir 50 through conduit 56 by pump 57 into hollow fiber cartridges 59. Fluid was allowed to flow until less than 1 liter of fluid remained in bioreactor chamber 28. At this point, the flow rate was increased so that all of the solution was transferred from bioreactor chamber 28 to hollow fiber reservoir 50 over

15 2 minutes. The nitrogen flow to bioreactor chamber 28 was then stopped, as was stirring within bioreactor chamber 28.

The microparticles were washed by pumping cold water (4°C) into the hollow fiber system through conduit 48 via conduits 41 and 42, using pump 46 at a flow rate of 80 ml/min. The volume of solution in the hollow fiber reservoir 50 was kept constant at 500 ml. Washing

20 was continued until 3.5 liters of cold water (4°C) had been pumped into the system. At this point, pump 46 was stopped. Pump 66 continued to carry out permeate at 80 ml/min until approximately 200 ml of solution remained in the hollow fiber reservoir 50. Pumps 46 and 57 were then stopped, and clamp 70 was opened to allow microparticles to flow from the hollow fiber reservoir 50 to beaker 72.

25 Clamp 70 was then closed and 100 ml of cold water (4°C) was introduced into the system from conduit 41 using pump 46, to rinse hollow fiber cartridges 59 so as to recover microparticles left behind in the first collection described above and to thereby maximize product yield. Pump 57 was then turned on, and the recirculating flow rate was slowly increased to 11 l/ml and maintained for 5 minutes, then decreased by gradually decreasing the flow rate, as

30 controlled by pump 57.

Clamp 70 was again opened to collect the microparticle suspension. The suspension was combined with the earlier collected microparticle suspension, and the weight of the combined microparticle suspensions was determined. The microparticles were stirred at room temperature for an additional time to further effect DCM removal.

5   The microparticle suspension was lyophilized after first aliquotting 1.5 ml into 5 ml vials. The vials were held at -50°C for 3 hours to freeze the suspension within the lyophilization chamber, after which the temperature was increased at the rate of 5°C per hour to a final temperature of -10°C. The vials were maintained at -10°C for a minimum of 6 minutes, and then at 25°C for 4-16 hours to lyophilize. The lyophilization chamber was backfilled with nitrogen

10   gas to a pressure of ~2" Hg, and rubber vial stoppers were seated while this pressure was maintained. When lyophilization was complete, the vials were crimped with rubber/aluminum septa, and stored at -20°C.

The particles were found to have a diameter of about 1-2 µm by number average.

EXAMPLE 3: Results

15   The procedure of Example 2 was carried out with various nucleic acid loadings and conditions for preparing the first emulsion. The results were as follows:

Trial #1

72 mg plasmid DNA
First emulsion prepared in microfluidizer
20   Yield: 4.6 g (77%)
Average microparticle diameter: 1.9 µm
Encapsulation efficiency: 4.8 µg DNA/mg microparticles
DNA remaining in supercoiled form: ≥60%
Residual PVA relative to the mass of microparticles: 0.88%

25

Trial #2

72 mg plasmid DNA
First emulsion prepared in SL2T homogenizer, batch process
Yield: 4.5 g (74%)
30   Average microparticle diameter: 2.1 µm
Encapsulation efficiency: 5.09 µg DNA/mg microparticles
DNA remaining in supercoiled form: ≥60%
Residual PVA relative to the mass of microparticles: 0.89%

Trial #3

72 mg plasmid DNA
First emulsion prepared in SL2T homogenizer (in this case, rather than 50 ml DCM and 10.8 ml
DNA solution, all 150 ml DCM and all DNA solution was combined at once)
5   Yield: 5.0 g (83%)
Average microparticle diameter: 2.0 μm
Encapsulation efficiency: 3.99 μg DNA/mg microparticles
DNA remaining in supercoiled form: ≥80%
Residual PVA relative to the mass of microparticles: 1.1%

10

Trial #4

108 mg plasmid DNA
First emulsion prepared in SL2T homogenizer
Yield: 5.6 g (93%)
15   Average microparticle diameter: 1.9 μm
Encapsulation efficiency: 7.04 μg DNA/mg microparticles
DNA remaining in supercoiled form: ≥60%
Residual PVA relative to the mass of microparticles: 1.48%

20   Trial #5

108 mg plasmid DNA
First emulsion prepared in SL2T homogenizer,
Yield: 5.75 g (96%)
Average microparticle diameter: 1.8 μm
25   Encapsulation efficiency: 9.78 μg DNA/mg microparticles
DNA remaining in supercoiled form: 70%
Residual PVA relative to the mass of microparticles: 1.63%

EXAMPLE 4: Alternative method for removal of residual organic solvent from the second
30   emulsion

The microparticles are formed as described in Example 2. The secondary emulsion is

pumped into a solvent removal device, wherein the DCM diffuses from the microparticles into

the aqueous phase of the secondary emulsion. The slurry is agitated very gently in this tank;

avoidance of vigorous agitation and aeration helps preclude the formation of foam. In contrast to

35   the procedure described in Example 2, there is no gas overlay, but rather a partial vacuum is

pulled over the headspace, venting any DCM that evaporates. As the DCM diffuses into the

aqueous phase from the microparticles, DCM levels rise in the aqueous phase until reaching

saturation (approximately 1-2%, dependent on temperature of the solution). During the

hardening phase, additional water is added to the solvent removal device until a total of 10 liters

of water is present per 150 ml DCM. The slurry is then transferred (e.g., by pressurizing the hardening tank) to a Sweco PharmaSep Filter/Dryer (Emerson Electric). This unit is fitted with a one-micron or less nominal screen size to retain microparticles greater than one micron in diameter. The screen size can be adjusted to retain microparticles of varying sizes. The product

5   is retained inside the filter/dryer as the aqueous phase passes through the screen and is discharged.

Inside the Sweco unit, the microparticles are then washed slowly with water for injection (WFI), keeping the microparticles in suspension through screen vibration. The DCM passes from the microparticles into the water, which passes through the unit and carries the DCM away.

10   Since the wash water entering the unit has no DCM, the microparticles are exposed only to very low DCM levels in the bulk aqueous phase, thereby speeding up the diffusive process for transferring DCM out of the microparticles. As DCM levels in the product drop, the flow rate of wash water can be reduced to conserve costs and aid solvent removal. The temperature of the wash water can be adjusted so as to maximize the rate of diffusive transfer without damaging the

15   product. Finally, the product can be rinsed with a final excipient and dried with nitrogen gas to the appropriate state of dryness. The dried product is discharged from the filter/dryer into a sterile receiving container for vialing. Alternatively, the product can be retained in the filter device for washing, and then dried in a lyophilizer prior to discharge into a sterile receiving container for vialing.

20   If desired, the DCM can be recovered from the discharged wash water by distillation. Since the discharged wash water contains no product, it can be boiled and the offgas passed into a distillation column. DCM and water can be separated on the distillation column and the DCM can be recovered for re-use. This has the benefits of reducing the cost of DCM, which is generally lost in the current processes, and of eliminating the environmental problems associated

25   with DCM vented to the atmosphere.

EXAMPLE 5: 2X scale of the microparticle production process.

The 2X scaleup of the process calls for two primary homogenizers, one secondary (flow-through) homogenizer, two bioreactors, and one (2x-sized) hollow-fiber device.

1. A single emulsion was made using 6 g PLGA in 50 ml DCM and 72 mg of DNA in

30   10.8 ml of 10% sucrose in a 250-ml polypropylene custom container sealed by a

            TEFLON® stopper through which the emulsifier head penetrates. After 15 minutes

of homogenization with a Silverson SR2T homogenizer at 10,000 rpm, an additional 100 ml of DCM was pumped into the container. The final volume of this emulsion was 161 ml. Homogenization proceeded for an additional 30 seconds.

2. Without removing the homogenizer tip, the emulsion was pumped out of the container through a stainless-steel tube that penetrated the TEFLON® stopper and reached the bottom of the container. The primary emulsion was pumped at 32.2 ml/min (5 minutes) along with 5 liters of PVA solution (1% PVA plus 10.37% sucrose), which was pumped at 1 liter/min (5 minutes) through a Silverson L4RT™ flow-through homogenizer into a 10-liter New Brunswick Scientific Bioflo™ reactor vessel. The reactor vessel was agitated at 375 rpm and sterile-filtered nitrogen gas was passed through the headspace at 20 liters/minute. The temperature of the reactor vessel was kept at 20°C for 30 minutes, then raised to 37°C and held at that temperature while continually stirring and gassing for an additional 2 to 3 hours.

3. While the first emulsion was stirring, step 1 was repeated with a second container and homogenizer.

4. Step 2 was repeated using the same secondary homogenizer and a different reactor vessel.

5. After the contents of first reactor vessel had stirred for 2 to 3 hours, the liquid in the reactor vessel was pumped at 8 l/min through a large hollow-fiber cartridge and recirculated back into the reactor vessel. The permeate was removed at a constant 160 ml/minute and discarded.

6. When one liter of permeate had been removed from the first reactor vessel, a set of valves was switched so as to put the second reactor vessel into the recirculation circuit. One liter of permeate was removed from the second reactor vessel and discarded.

7. Steps 5 and 6 were repeated until the volume of suspension in each vessel was one liter. At that point, the contents of both reactors were pumped into a two-liter reservoir adjacent to the hollow-fiber apparatus and were recirculated through the hollow-fiber device at 8 liters per minute. Permeate is continuously removed at 160 ml/min.

8. When the volume of the suspension was one liter, WFI was pumped into the reservoir

at 160 ml/min. Permeate was continuously removed at 160 ml/min. The flow of WFI was stopped when seven liters had passed through the system.

9. The recirculation flow was reduced to 4 liters/minute and permeate flow was reduced to 80 ml/min. The pumping continues until the volume of the suspension is about 400 ml.

10. The pumps are stopped at this time and the suspension was drained from the hollow fiber apparatus into a sterile receiving vessel.

11. A concentrated solution of excipient was added to the product at this stage. The product was allowed to stir for 5 minutes.

12. The product was dispensed into vials for lyophilization, stoppering, and crimping.

Results: The microparticles were analyzed and the following results were obtained:

Encapsulation (determined by UV absorption): 4.0 µg DNA/mg particles

Supercoiling: 60% in microparticles

Microparticle diameter: 2.0 µm by number average, 6.2 µm by volume average; 80% were larger than 2.8 µm in diameter

Yield: 10.3 g (85.8%) in 650 ml water

PVA concentration: 1.1%

EXAMPLE 6: 10X and up scale for microparticle production

The following describes the technique for production of microparticles at the 60-gram and higher scale.

1. An aqueous solution of DNA and sucrose, and a solution of PLGA and lipid in DCM, are simultaneously pumped through a continuous flow homogenizer to form the primary emulsion.

2. The primary emulsion and a solution of PVA/sucrose are simultaneously pumped through a second continuous flow homogenizer to form the secondary emulsion. The second emulsion is passed into a solvent removal device, forming a suspension of microparticles.

3. The suspension is pumped into a screen-type washer-dryer (e.g., a Sweco device), in which the product is washed with a first aqueous solution or WFI. This is followed by a washing with a second aqueous solution that contains an excipient (e.g.,

mannitol).

4. The product is dried inside the Sweco unit by a steady stream of dry, sterile air.

5. The dried product is removed from the Sweco unit through the discharge port into a sterile receiving vessel.

5   6. The dried product is filled into vials with a powder auger. This screw-like device raises the powder from the receiving vessel and dispenses it into injection vials that are then capped and crimped.